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Preservation of the Leukemogenic Activity and Elimination of Contaminants During Purification of Rauscher Virus

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INTRODUCTION

SEVERAL methods have been applied to the purification of murine leukemia viruses and particularly to the purification of Rauscher virus. One of the first to be used was differential centrifugation in a potassium citrate buffer [1]. Unfortunately it allows only a partial purification and it is accompanied by a noticeable inactivation of the biological activity of the virus as shown by Ziegel and Rauscher [2] and ourselves [3]. Centrifugation in a density gradient in salt solutions such as Caesium chloride [4, 5] Rubidium chloride [5] potassium citrate and potassium tartrate [5] increases the purity of the virus, but does not prevent its partial inactivation. Centrifugation in a density gradient in non ionic solutions such as polysucrose (Ficoll) [6], polyglucose [6], sucrose [7] seems to be the best method for the preservation of the biological activity of the virus during the purification process.

We purified the Rauscher virus by means of an ultracentrifugation in sucrose [8], followed by centrifugation in a sucrose gradient.

The aim of this work was to determine the fate of the leukemogenic activity of the virus during the course of this purification procedure, and to determine the degree of purification obtained by comparison with other techniques such as precipitation by ammonium sulfate, differential centrifugation in a citrate buffer, centrifugation in a Ficoll gradient.

MATERIAL AND METHODS

(A) Virus source

RV was obtained from two sources:

— *Tissue culture grown RV (CRV)*. Monolayers of JLS V5 cell line [9, 10], were obtained in casein hydrolysate medium supplemented with 15% calf serum. The supernatant, rich in virus particles, was collected approximately 44 hr after the initiation of the culture. Labeled virus was produced by adding phosphate-free casein hydrolysate medium containing 5 μ c of $H_3P^{32}O_4$ per milliliter on to a growing JLS V5 cell layer; the supernatant was collected as above.

— *Mouse plasma RV (MRV)*. One month old BALB/c mice were inoculated intraperitoneally with 0.1 ml of a RV suspension (average titer: 10^4 to 10^5 ED₅₀/ml) and sacrificed 4-5 weeks later. The blood was collected and mixed with a negligible volume of heparin solution in distilled water. Final heparin concentration was 10 to 15 i.u. per milliliter of blood.

(B) Description of the purification procedure

It included 3 steps, all carried out at + 4°C:

(1) *Clarification*. Tissue culture supernatant or plasma was first clarified by 3 successive low speed centrifugations: 1200 g for 20 min; 2400 g for 20 min; 10,000 g during 2 min. The supernatant fluid was collected each time.

(2) *Ultracentrifugation in 0.25 M sucrose*. The clarified virus suspension was added to an equal volume of a solution containing 0.5 M purified

sucrose buffered with 15×10^{-4} M Tris-HCl, pH 7.2. This mixture was then centrifuged for 90 min at 56,000 g and the virus pellet was resuspended with a teflon pestle homogenizer in 0.5 to 1 ml of a 0.025 M sucrose solution buffered in the same way, i.e. about one hundredth of the initial volume.

(3) *Rate-zonal centrifugation in sucrose.* A 5–20% w/v sucrose gradient (in Tris-HCl) was performed over 4.5 ml of a 40% sucrose solution; the virus suspension from step 2 was immediately layered and centrifuged 15–40 min at 25,000 rev/min in a Spinco SW 25 rotor. Approximately 60 fractions were collected by puncturing the bottom of the tube.

(C) *Other techniques of purification assayed for comparison*

(1) *Precipitation by ammonium sulfate.* This technique can be performed only when crude RV has been previously cleared of most of the surrounding proteins.

When using CRV, a casein hydrolysate medium supplemented with 0.5% calf serum was added in replacement of the growth medium 20 hr before collection. The supernatant was concentrated ten times in Visking cellophane tubes by dialysis against polyvinyl pyrrolidone. This concentrated fluid was precipitated with an equal volume of saturated $(\text{NH}_4)_2\text{SO}_4$ solution neutralized with NH_4OH at 0°. The precipitate was collected by low speed centrifugation and resuspended in 1–5 ml of sodium citrate buffer 0.05 M at pH 6.8.

When using MRV, the plasma was filtrated through a 60×5 cm Sephadex G 200 column, the eluent was sodium citrate buffer 0.05 M at pH 6.8. The virus was, as demonstrated by a bioassay, eluted with the external volume of the column after 15 to 20 hr at +4°C, in a volume equal to the initial volume of plasma and was cleared of 80–85% of the plasma proteins. It was subsequently precipitated by ammonium sulfate.

(2) *Differential centrifugation in potassium citrate* 0.153 M. This was performed according to the technique described by Moloney [1].

(3) *Rate-zonal centrifugation in ficoll.* The technique of Oroszlan *et al.* [6] was used.

(D) *Criteria of purification*

(1) *Radioactive contaminants.* For practical convenience, the radioactive contaminants were prepared from cell cultures *in vitro* and added to the CRV.

The principle of this method is based on the assumption that contaminants surrounding CRV are not specific of JLS V5 supernatants but similar to those present in supernatants of other cell cultures. The supernatant of un-

labeled JLS V5 cell culture was mixed with the supernatant of mouse embryo fibroblasts (MEF) labeled with P^{32} for a long period of time in order to label all the contaminants. Purification of the mixture was achieved as previously described. The specific activity was determined at each purification step. If one assumes that labeled MEF and unlabeled JLS V5 contaminants are equally eliminated, a decrease in specific activity corresponds to a preferential loss of contaminants as compared to loss of the unlabeled virus pool and assesses the value of the purification procedure.

MEF were prepared in the same way as JLS V5 cell cultures, except that the medium was phosphate-free and contained $10\mu\text{C}$ of $\text{H}_3\text{P}^{32}\text{O}_4$ per milliliter. Three to four days after completion of the monolayers, the culture fluid was collected and centrifuged twice at 2000 g during 5 min. The supernatant containing the "particulate contaminants" was mixed with an equal volume of unlabeled JLS V5 culture supernatant. The purification was conducted as previously; at each step, aliquots were taken and measured for radioactivity in a Tricarb liquid scintillator. In order to determine the specific activity, the phosphorus content of each sample was determined by the Horecker [11] method adapted to a micro-method in our laboratory.

In addition, to evaluate the capacity of adsorption of mineral phosphates ("soluble contaminants") on the virus, a Tris casein hydrolysate medium plus $10\mu\text{C}/\text{ml}$ of $\text{H}_3\text{P}^{32}\text{O}_4$ was mixed with JLS V5 culture supernatant or leukemic mouse plasma and the purification was conducted as previously described.

(2) *Titration of RV leukemogenic capacity.* Bioassays were performed by the spleen-weight method [2, 10]. The infective titer ($\text{ED}_{50}/0.1$ ml) was calculated by the Reed-Muench method. The standard deviation of this method was ± 0.25 to 0.33 log. Titrations were made at each step of purification on the various virus samples diluted to the initial volume of plasma. Titrations were exclusively performed with mouse plasma, since the infectivity of the cultured virus is very low [12].

(3) *Electron microscopy.* The negative staining technique of Brenner and Horne [13] was employed for detecting viruses in each sample examined.

RESULTS

Purification of virus

Rate-zonal centrifugation in sucrose, performed with CRV or MRV previously clarified and ultracentrifuged, showed two bands after

15 min of centrifugation: an upper band with a lower sedimentation rate separated from a bottom band which was located at the inter-phase between the 40% sucrose solution and the 20–5% w/v density gradient.

These 2 bands were visible to the naked eye when a sufficient amount of virus was employed. Their position was also determined by radioactivity with labeled CRV (Fig. 1).

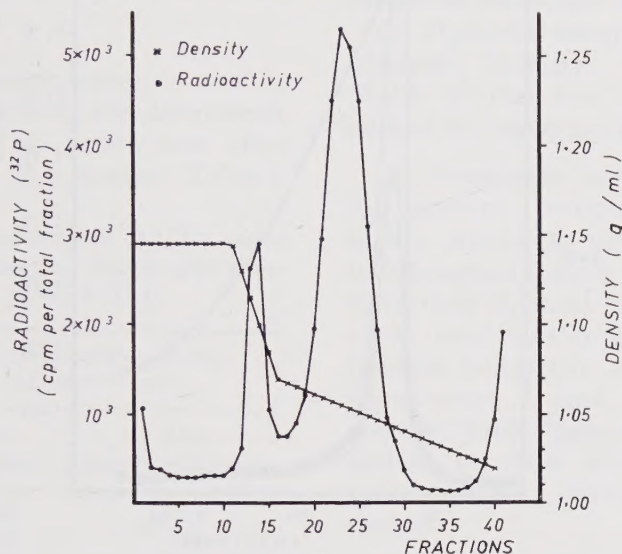


Fig. 1. Rate-zonal centrifugation in sucrose of P^{32} labeled RV extracted from JLS V5 cell culture supernatants and concentrated by ultra centrifugation in sucrose 0.25 M. Centrifugation in SW 25 rotor at 25,000 rev/min for 15 min.

When the centrifugation time was extended to 40 min, both bands merged into the inter-phase (Fig. 2).

There was also some material close to the

CRV and MRV gave identical profiles, sedimentation rate and buoyant density.

Elimination of radioactive contaminants

During the two first steps of the purification

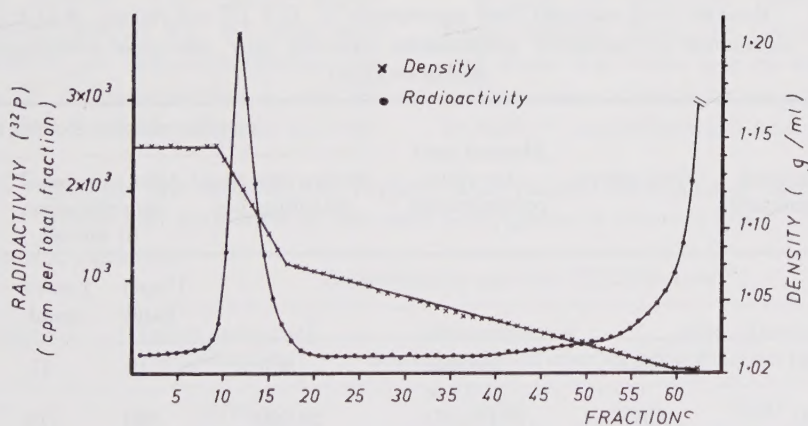


Fig. 2. Rate-zonal centrifugation in sucrose of P^{32} labeled RV extracted from JLS V5 cell culture supernatants and concentrated by ultra centrifugation in sucrose 0.25 M. Centrifugation in SW 25 rotor at 25,000 rev/min for 40 min.

meniscus which has not been thoroughly investigated and which showed no tendency to migrate.

After dialysis, the two sucrose bands were examined in the electron microscope; morpho-

procedure, most of the contaminants were eliminated, as appreciated by electron microscopy. For that reason measures concerning the elimination of radioactive contaminating material were done only after the second step;

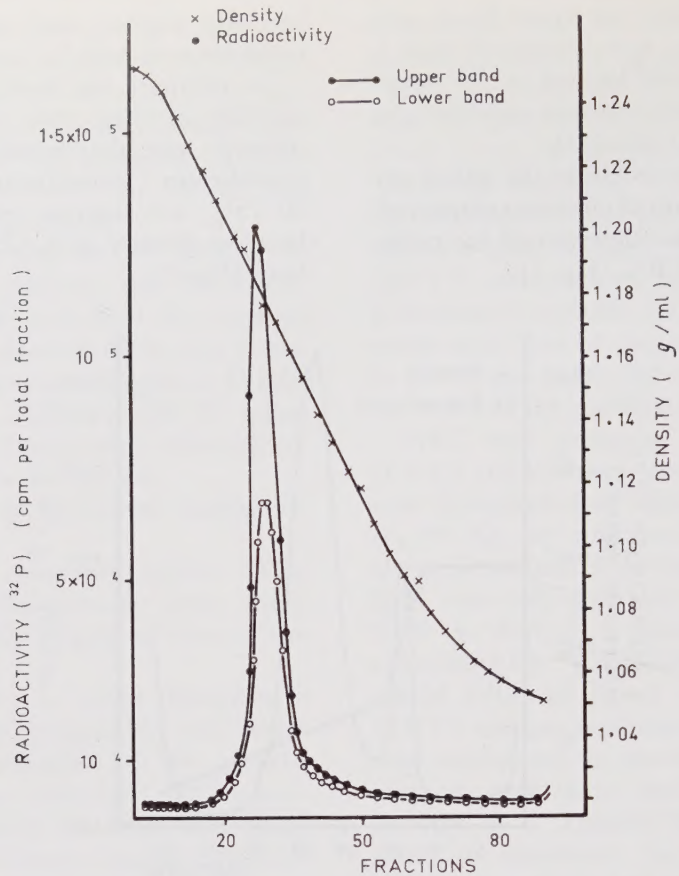


Fig. 3. Relayering of the dialyzed sucrose bands of P^{32} labeled RV in a 60–15 % w/v sucrose density gradient in Tris-HCl and centrifugation at equilibrium (20 hr at 33,000 rev/min in SW 39 Spinco rotor).

so that the value of the criteria for the purification procedure was thus more severe, since we took as 100 the percentage of radioactivity remaining after this step.

Table 1 (experiments 1 and 4) shows that

after rate-zonal centrifugation in sucrose the specific activity of the mixture (unlabeled CRV plus labeled contaminants) markedly decreased indicating that 95–98% of the contaminating material present at step 2 of purification

Table 1. Rauscher virus extracted from supernatants of JLS V5 cell cultures BALB/c mouse plasma: elimination of radioactive contaminants occurring after rate-zonal centrifugation in sucrose or Ficoll

Experiments	P ³² labeled contaminants*	Virus source	Method used for virus concentration	Specific activity (counts/min µg P)			
				Before rate-zonal centrifugation	After rate-zonal centrifugation in sucrose		After rate-zonal centrifugation in Ficoll†
					Upper band	Lower band	
1	SC	CRV	sucrose 0·25 M	950	19	47	
2	SC	CRV	(NH4) ₂ SO ₄	20,000	580	570	
3	SC	MRV	sucrose 0·25 M	1,430	57	71	
4	PC	CRV	sucrose 0·25 M	17,000	450	500	700
5	PC	CRV	(NH4) ₂ SO ₄	45,000	1580	3150	1600

*Soluble contaminants (SC), particulate contaminants (PC). For details concerning the contaminants, see material and methods.
†Only the middle band was assayed.

was eliminated. In addition a similar decrease in specific activity occurred whatever "particulate contaminants" or "soluble contaminants" were used. Therefore it may be suggested that the residual contamination was due to an adsorption of small molecules on to the virus membrane, probably adsorbed from the very first purification step. Experiment number 3 indicates that elimination of "soluble contaminants" with MRV is similar to that obtained with CRV.

Appreciation of the leukemogenic potency

Virus leukemogenic activity was determined with infectious MRV (a) before and after ultracentrifugation in 0.25 M sucrose: Table 2

Table 2. *Rauscher virus extracted from BALB/c mouse plasma: conservation of leukemogenic activity after ultracentrifugation in sucrose 0.25 M*

Experiment	Leukemogenic activity (ED 50/0.1 ml)*	
	After clarification	After ultracentrifugation
1	3900	2800
2	4600	5000
3	10,000	10,000

*The volume of each sample submitted to titration was previously adjusted to the original plasma volume with a 0.025 M sucrose solution buffered with 15×10^{-4} Tris-HCl, pH 7.2.

shows that there was no loss of activity during this step. (b) before and after rate-zonal centrifugation in sucrose. The unique band formed after centrifugation for 40 min was titrated. The results of this experiment are presented in Table 3 (experiments 1 and 2) and demonstrate that the leukemogenic activity

of RV remains practically unimpaired. Moreover, the two sucrose bands obtained after 15 min of centrifugation were also assayed separately for infectivity. The results of two experiments demonstrate that nearly all the leukemogenic activity was located in the upper band: 1000 and 3150 ED 50/0.1 ml in the upper band as compared to 3 and 1.5 ED 50/0.1 ml in the lower band.

Comparison with results obtained by other techniques

(a) *Differential centrifugation in potassium citrate (Moloney's technique)*. Bioassay demonstrated (Table 4) that this technique strongly diminished the leukemogenic activity of MRV.

(b) *Precipitation with ammonium sulfate*. On the contrary, precipitation with ammonium sulfate proved to conserve satisfactorily the leukemogenic activity of MRV (Table 4). The virus thus obtained was submitted to a rate-zonal centrifugation in sucrose and tested for residual radioactive contamination (Table 1, experiments 2 and 5) and leukemogenic activity (Table 3, experiment 3). Results were similar to those obtained when RV was concentrated by ultracentrifugation in 0.25 M sucrose.

(c) *Rate-zonal centrifugation in Ficoll*. RV previously ultracentrifuged in 0.25 M sucrose or precipitated with ammonium sulfate was submitted to a rate-zonal centrifugation in Ficoll and sedimented in three bands, as described by Oroszlan *et al.* [6]. The complete viral particles were concentrated in the middle band as shown by electron microscopy.

Results of titrations performed with the Ficoll middle band are presented in Table 3 and show that there was no loss of activity.

The value of residual contamination is given in table 1 (experiments 4 and 5). The decrease

Table 3. *Rauscher virus extracted from BALB/c mouse plasma: conservation of leukemogenic activity after purification by rate-zonal centrifugation in sucrose or Ficoll*

Experiment	Leukemogenic activity (ED 50/0.1 ml)*		
	Before rate-zonal centrifugation†	After rate-zonal centrifugation in sucrose‡	After rate-zonal centrifugation in Ficoll§
1	5750	5500	5600
2	10,000	10,000	10,000
3	3170	3200	3200

*The volume of each sample submitted to titration was previously adjusted to the original plasma volume with a 0.025 M sucrose solution buffered with 15×10^{-4} Tris-HCl, pH 7.2.

†Experiments 1 and 2 relate to virus concentrated by ultracentrifugation in 0.25 M sucrose. Experiment 3 relates to virus precipitated by ammonium sulfate.

‡Centrifugation for 40 min at 25,000 rpm.

§Titration relates to the middle band alone.

Table 4. Rauscher virus extracted from BALB/c mouse plasma: leukemogenic activity after precipitation by ammonium sulfate or ultracentrifugation in potassium citrate

Experiment	Leukemogenic activity (ED ₅₀ /0.1 ml*)	
	Clarified plasma	The same after gel filtration on sephadex and precipitation by ammonium sulfate
1	300	270
2	10,000	9900
3	1100	1000
		The same after ultracentrifugation (90 min at 56,000 g)
4	720	45
5	1500	70
6	4800	800
7	4800	750
8	13,500	1260
9	1200	75

*The volume of each sample submitted to titration was previously adjusted to the original plasma volume with sodium citrate buffer, 0.05 M, pH 6.8.

in specific activity was about the same as that obtained after rate centrifugation in sucrose.

DISCUSSION

The purification of Rauscher virus in sucrose allows the preservation of nearly the entire biological activity of the virus. It discards the major part (95–98%) of cellular contaminants labeled with P³² and attached to the viral particle.

It should be noted that in the present study, the biological activity was determined on MRV and the elimination of contaminants appreciated on CRV. However, it seems reasonable to extend to MRV the results which were obtained with CRV and *vice versa*, since, except for the leukemogenic potential, the identity of RV from both *in vivo* and *in vitro* sources, has been demonstrated by several reports, dealing with the morphology in the electron microscope [2, 12], the buoyant density in CsCl [4] and in sucrose (present work) the sedimentation rate (present work), and the characteristics of the RNA [7].

The value of the method of purification of Rauscher virus in a sucrose solution, which was already emphasized by Oroszlan *et al.* [6] and Duesberg *et al.* [7] is fully confirmed by the present study.

The other purification methods seem to be less satisfactory.

Differential centrifugation in potassium citrate according to Moloney's method inactivates most of the virus. This inactivation seems to result from the virus concentration by high speed centrifugation in salt solutions, for it is not observed when the virus is exposed to potassium citrate without ultracentrifugation [15], whereas ultracentrifugation in non ionic solutions does not significantly affect the virus infectivity.

Precipitation by ammonium sulfate, although conserving entirely the leukemogenic activity of the virus, has some drawbacks, since it needs long and elaborate operations due to the fact that it requires a virus suspension poor in proteins which would otherwise precipitate with the virus. Rate-zonal centrifugation in Ficoll brings about a satisfactory purification of RV, as already emphasized by Oroszlan *et al.* [6] but, in our opinion, appears less convenient than sucrose gradient: it cannot be submitted to dialysis and the virus band obtained cannot be examined directly in the electron microscope unless it is diluted and recentrifuged again in order to obtain a viral pellet free of polymer. Moreover, Ficoll solutions adsorb u.v. light waves and prevent optical density measurements. In addition, it can be pointed out that sucrose gradient gives a sharp resolution of the RV bands in a shorter period of time, since 15 min is sufficient to obtain a good separation of the active virus particles.

An interesting data brought about in the present study is that the two virus bands obtained by rate centrifugation in sucrose contain a similar concentration of apparently intact virions when examined in the electron microscope. However according to the bio-assay, the upper virus band alone is infective. With respect to this technique, infective and non infective virus particles would have different sedimentation rates. On the contrary, they occupy a single position after equilibrium centrifugation in sucrose. The density of the two virus populations being the same, the particles may differ with regard to size or shape. Further investigations are necessary to clear up that fact.

Concerning the virus purity test, one can assume that radioactive contamination on the virus band was probably due to an adsorption of mineral phosphates into the virus, since the percentage of residual contamination persisting after the last purification step was almost the same when "particulate contaminants" or "soluble contaminants" were used. It appears

that complete RV purification cannot be achieved by the usual centrifugation technique. Other methods based on different principles, such as electrophoresis, may be useful for solving this problem.

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RESUME

Le virus leucémique de Rauscher, extrait du plasma de souris leucémiques BALB/c ou de surnageant de la lignée JLS V5 chroniquement infectée par ce virus, a été purifié par trois centrifugations à faible vitesse, suivies par une ultra-centrifugation en sucrose 0,25 M, et finalement par une centrifugation en gradient de sucrose. A la suite de ces opérations, le pouvoir leucémogène du virus était presque entièrement préservé (perte de 1 à 2%), et environ 95–98% des contaminants cellulaires marqués par le ^{32}P étaient éliminés. Des deux bandes virales obtenues dans le gradient sucrose, la supérieure seule s'est montrée infectieuse. La contamination résiduelle a été prouvée comme due principalement à une adsorption sur la coque virale de phosphates minéraux.

La valeur de cette méthode a été comparée à celle d'autres procédés de purification (centrifugation différentielle en citrate de potassium, précipitation par le sulfate d'ammonium, centrifugation en gradient de Ficoll).

SUMMARY

Rauscher murine leukemia virus (RV) extracted from leukemic BALB/c mouse plasma or JLS V5 cell culture supernatants was purified by three low speed centrifugations, followed by ultracentrifugation in sucrose 0.25 M and finally rate-zonal centrifugation in sucrose. At the end of this purification procedure, the leukemogenic activity was almost entirely preserved (loss of 1 to 2%) and about 95–98% of the radioactive contaminants were eliminated. Two viral bands were obtained by rate centrifugation in sucrose; the upper one was only infective. The residual contamination was proved to be mainly due to an adsorption of mineral phosphates.

The value of this method was compared with other purification procedures (differential centrifugation in potassium citrate, precipitation by ammonium sulfate, rate-zonal centrifugation in Ficoll).

ZUSAMMENFASSUNG

Aus leukämischem BALB/c Mausplasma oder aus JLS V5-Zellkulturüberstand extrahiertes Rauschersches Mäuseleukämievirus wurde durch dreimaliges Zentrifugieren bei niedriger Drehzahl gereinigt. Es folgte eine Ultrazentrifugation in Sucrose 0,25 M und schliesslich zonales Sedimentieren in einem Sucrose-gradienten. Im Anschluss an diese Reinigung war die leukämogene Aktivität noch fast vollständig erhalten (nur 1–2% Verlust); 95–98% der radioaktiven Verunreinigungen waren eliminiert. Durch die zonale Zentrifugation in Sucrose wurden zwei virale Streifen erhalten, wovon nur der obere infektiös war. Die verbleibenden Verunreinigungen bestanden hauptsächlich aus einer Absorption mineraler Phosphate.

Der Wert dieser Methode wurde mit anderen Reinigungsverfahren verglichen (Differenzialzentrifugieren in Kaliumzitratlösung, Ausfällung durch Ammoniumsulfat, zonales Zentrifugieren in Ficoll Lösung).

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